Optimal Durations of Single Exposures to a Frequency-Modulated Magnetic Field Immediately After Bisection in Planarian Predict Final Growth Values

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Planarian (*Dugesia tigrinia*) were exposed to a frequency-modulated ("Thomas"), patterned electromagnetic field (EMF) immediately following transection through the pharynx. Subjects were exposed from 15 min to 3 h as well as single versus repeated exposures. Results from multiple experiments indicated that those planaria exposed from 45 to 90 min regenerated at significantly higher rates than those exposed less than 45 min. In addition, the study revealed that exposures greater than 45 min were not significantly different beyond this inflection point. We suggest that this particular pattern of EMF is capable of inducing biochemical pathways associated with cell proliferation, in particular the p38-MAPK and hsp70 pathways. Bioelectromagnetics 9999:1–5, 2013. © 2013 Wiley Periodicals, Inc.

Key words: patterned magnetic fields; electromagnetic fields; planaria; p38; MAPK; regeneration

INTRODUCTION

The sensitivity of flatworms, planarian, to weak static magnetic fields was reported several decades ago by Brown [1975] and Brown and Chow [1962]. These remarkable invertebrates, who can reproduce asexually as well as sexually, display a disproportionately large population (20–30% of all cells) of neoblasts that can potentially differentiate into many types of adult cells [Agata et al., 1998; Newmark and Alvarado, 2002; Reddien and Alvarado, 2004]. The regenerative ability of the planarian is reflected in the capacity for as little as 1/279 of a planarian [Lobo et al., 2012] to differentiate into a new worm within 1–2 weeks. The biomolecular mechanisms associated with regeneration as well as the calcium and potassium channel changes that control regrowth have been delineated [Beane et al., 2011].

That weak, extremely low frequency magnetic fields, in the order of tens and hundreds of nT, can affect the rate of regeneration in planarian has been reported by Novikov et al. [2008]. Different amplitudes (as low as 40 nT) and frequencies (1, 3, 7, 32, and 60 Hz) exhibited stimulatory capacity while others did not. Madkan et al. [2009] also reported differential rates for growth, depending upon the employed frequencies (8, 16, and 72 Hz) in planarian exposed after transection to nanosecond pulsing electromagnetic fields (EMFs) for 1 h daily. There was a general increase in heat shock protein (hsp) 70 in the tail portions of the segments when they were exposed to any of the three frequencies and durations between 20 and 80 min [Madkan et al., 2009]. Goodman et al. [2009] confirmed that exposure for 1 h twice per day to 60 Hz, 8 μT magnetic fields during the initial 3 days post-surgery caused increased regeneration. The effect was more pronounced for tails and was associated with increased hsp70 protein levels and activation of the extracellular regulated signal kinases.

Magnetic configurations tuned for calcium resonance [Tiras et al., 1996] increased regeneration following exposures between 4 and 240 h. On the other hand Jenrow et al. [1995] found that 23 h exposures per day to a combined direct current

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(78.4 μT) and alternating current (60 Hz at 10 μT), tuned to calcium parametric resonance, significantly delayed regeneration of cephalic sections that was dependent upon the orientation of the planarian to the field vectors. These observations are consistent with the likely contribution of calcium signaling to membrane voltage-mediated changes in gene expression within the anterior head section [Beane et al., 2011].

One significant question is: what is the shortest duration required for magnetic field exposure following bisection that results in most of the fastest regeneration? In other words, what is the inflection point for the relationship between the duration of *single* exposure to magnetic field after surgery and the maximum rate of growth or final growth? The shape of this relationship as well as the absolute temporal value required to achieve this asymptote may be revealing for pursuing the plausible mechanism.

In the present study we exposed freshly sectioned planarian to intervals between 0 and 3 h to a complex, pulsed frequency-modulated magnetic field ("Thomas pulse") that has been shown to affect analgesic thresholds or cell-relevant reactions in variety of invertebrates and vertebrates [Thomas et al., 1997; Martin et al., 2004]. A single exposure for about 45 min is sufficient to produce an effect size (the amount of variance explained by the treatment) that is at least two-thirds of the maximum effect size.

MATERIALS AND METHODS

Planaria Cultures

Stocks of living brown planaria (*Dugesia tigrina*) were obtained from Carolina Biological Supply (Burlington, NC). Upon arrival, specimens were divided into groups of approximately 100 and placed within plastic culturing containers measuring 23 cm by 36 cm. Water used for the cultures was from a natural spring source (Grey County, Ontario) and the containers were housed in complete darkness for approximately 1 week before any testing began. Raw beef liver was added to each container every week for 1–2 h to permit feeding, after which the culture water was changed to dispose of waste. Planaria used for the experiment were food deprived for at least 7 days prior to test initiation. Temperature conditions were maintained at room temperature ($25 \pm 2\ ^{\circ}C$) both prior to and during the experiment.

Lesion and Regeneration Assay

Subjects were taken directly from the culture containers and placed within a 23 cm by 23 cm clear Pyrex dish filled with natural spring water and superimposed upon a 5 mm by 5 mm grid. A single light served to both illuminate the cutting platform and direct the planaria by phototaxis away from the light. When a subject was fully extended, a single bisecting cut was completed with a fresh scalpel blade directly through the pharynx. Photographs were taken of head and tail sections. Lengths were determined by comparing worm distention to the background grid. Net increase in length was calculated as (length day X) − (length day 0) for each worm.

In total, each experimental group consisted of 5 head and 5 tail sections. Rate of regeneration was measured by photographs taken every second day (Days 1, 3, 5, and 7) at approximately the same time as the initial bisection. Photographs were analyzed using ImageJ 1.46 available from the National Institutes of Health website (Bethesda, MD).

Magnetic Field Exposure

Test subjects were housed in 250 ml plastic cups with 25 ml of natural spring water per 5 heads and tails. The entire test group, with the exclusion of the sham exposures, was placed directly within the magnetic field generator. The generator was a 30 Ω Helmholtz coil comprised of a 39 cm by 39 cm box wrapped by 305 m of 30-gauge AWG insulated copper wire.

The pattern was generated by converting a row of 849 numbers between 0 and 255 into voltages. Values above 127 were positive polarity while values below 127 were negative polarity between −5 V and +5 V using a Zenith-386 computer (Model ZF-148-41, Zenith Data Systems, Benton Harbor, MI). A digital to analogue converter was utilized along with specialized software to convert the numbers to the appropriate current necessary to generate the field. Both the software and digital-to-analogue converter were custom built by our technologist Stanley Koren.

The "Thomas" field itself is a frequency-modulated pattern that is characterized during the first 200 ms of 3 ms pulses at 25 Hz (Fig. 1). Following this there is a stepwise reduction down to 6 Hz within the last 500 ms. The value for each point was presented for 3 ms so that 2.55 s was required for the completion of each pattern. The time between generations of the pattern was 3 ms. Consequently the interstimulus (interpattern) interval was equivalent to the point duration. Stated alternatively, the presentation was continuous. The field had an average intensity at the center of the coil of 38 mG (range of 21.4–54.5 mG). Planaria not receiving the magnetic field (shams) were placed in an identical non-active Helmholtz coil for approximately 60 min.

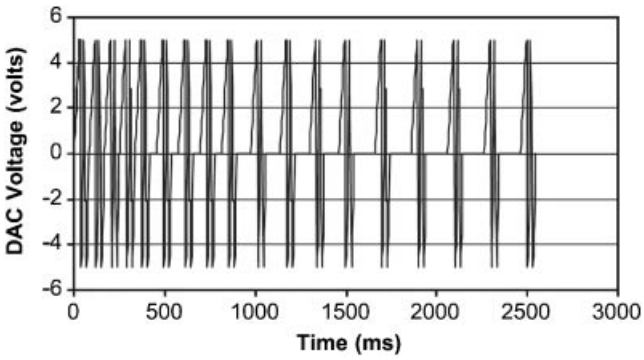

Fig. 1. Temporal structure of the "Thomas" pattern magnetic field employed in this study. The vertical axis refers to the voltage values converted from numbers between 0 and 255 (127 = 0 V). The horizontal axis reflects the temporal organization of the pattern created by the series of numbers. The duration of each number or point was 3 ms.

The experiment was conducted in three phases: short durations, long durations, and repeated exposures. In the short duration exposures, planaria were exposed for 0, 15, 30, 45, and 60 min to the magnetic field. Longer durations consisted of 0, 30, 60, 90, 120, and 180 min exposures. Finally, the repeated exposures utilized a 0, 30, 60, and 90 min duration schedule and were exposed once per day for the entire 7 days of the experiment. All experiments were conducted in triplicate.

Statistical Analysis

Analyses of variance and polynomial trends were completed as a function of days post-dissection, section of planaria (head vs. tail) and duration of magnetic field exposure. Post-hoc tests involved combinations of Tukey's (post hoc test). All analyses were completed with statistical product and service solutions (SPSS) software (IBM, Armonk, NY).

RESULTS

Three-way analysis of variance as a function of days post-dissection, cut (tail vs. head) and duration for the length of the worms demonstrated a statistically significant difference between durations of exposure ($F(7,50) = 3.28$, $P < 0.01$, $\eta^2 = 31\%$) but no significant interaction between head/tail and duration of exposure ($F(7,50) = 0.51$, $P > 0.05$). As expected there was a significant difference in length as a function of post-dissection day ($F(4,200) = 75.49$, $P < 0.001$, partial $\eta^2 = 0.60$). Analyses incorporating the repeated exposures showed no statistical significance between exposure durations ($F(7,50) < 0.1$, $P > 0.05$), with a significant difference between those planaria receiving a single exposure and a repeated exposure ($F(4,328) = 6.41$, $P < 0.001$, partial $\eta^2 = 0.08$).

Polynomial analysis (all degrees of freedom = 1.58) showed a statistically significant linear trend for this increase in growth as a function of the initial single duration of field exposure two ($F = 12.89$, $P < 0.001$), three ($F = 25.26$, $P < 0.001$), and four ($F = 16.14$, $P < 0.001$) days after the dissection. Particularly revealing was the statistically significant ($F(28,200) = 2.51$, $P < 0.001$, $\eta^2 = 26\%$) interaction between duration of field exposure and growth (in days). The major source of the interaction was the increased magnitude of difference between days 1 and 3 postsurgery after the single 45 min exposure compared to other exposures, particularly the no-field exposures (Fig. 2).

In order to display the effect sizes of the differences between net changes in growth on days 1, 2, and 3 after the surgery, ω^2 estimates were calculated for each of the eight different groups of single exposure times (0, 15, 30, 45, 60, 90, 120, and 180 min). As shown in Figure 3, the best fit curve indicated that the inflection for the explained variance (effect size) in growth occurred with the single exposure of approximately 30 and 45 min.

DISCUSSION

Several experiments have shown that exposures after surgical sectioning of planarian to weak, extremely low frequency magnetic fields facilitate regenera-

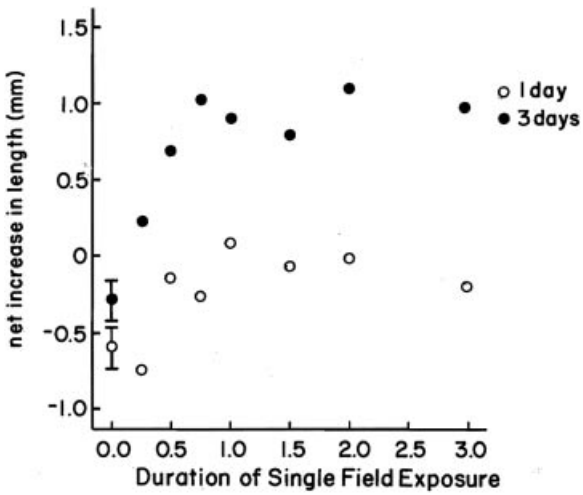

Fig. 2. Net increase in length (in mm) compared to baseline of planarian following various single durations of magnetic field exposures (horizontal axis in h) 1 and 3 days after the field treatment. Vertical bars indicate sample standard errors of the mean.

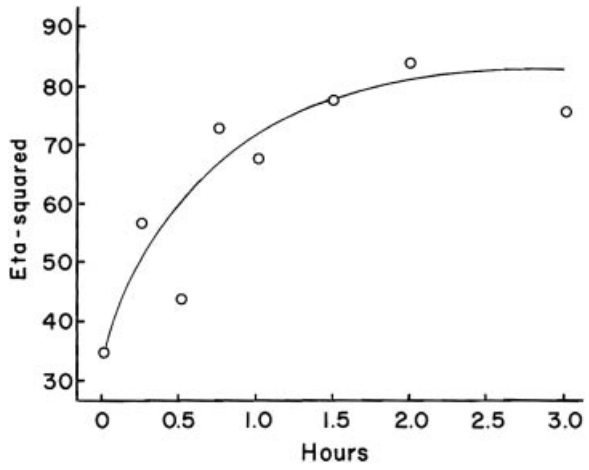

Fig. 3. η^2 estimate or amount of variance explained in net change of increase in growth during the experiment as a function of the single duration of exposure (in h) to the frequency-modulated ("Thomas") weak magnetic field.

tion. The durations of these exposures have ranged from an hour per day to near continuous exposures for over 100 h. The results of the present study indicate that the display of Wilder's Law (the point of diminishing returns or asymptote) for this particular model of sectioning and regeneration involved only a single, approximately 30–45 min exposure to the frequency-modulated magnetic field. Durations more than 45 min and up to 3 h did not appreciably increase the final regeneration measures 3 days later.

This observation, in addition to facilitating efficiency and parsimony for use of equipment and experimental time, indicates that the duration between 30 and 45 min can produce most of the effects produced by 3 h of exposure upon the ultimate regeneration or growth 3 days later. Thus the energy associated with this pattern of exposure would appear to affect a cellular process that only requires this duration for stimulation. Regeneration is initiated by closure of the wound within about 30–45 min [Lobo et al., 2012] and is facilitated by muscular contraction through molecular mechanisms that are unknown. Migration of planarian epithelium cells appears to be essential for wound closure that is followed by a transorganismic peak in cell mitosis from neoblasts within 6 h after the injury.

The metabolic pathway responsible for planarian regeneration was previously delineated by Beane et al. [2011], in which it was indicated that those pathways associated with regeneration were also intimately connected to those involving calcium and potassium channel changes. Previous work utilizing B-16 melanoma cells by Buckner [2011] indicate that 60 min exposures of the "Thomas" EMF induced a massive increase in intracellular calcium levels. It is possible that the metabolic pathways enabling planarian regeneration are similar, if not analogous to those pathways in the B-16 cells which result in the calcium influx, and possibly connected to the increased cellular proliferation rate associated with malignancies.

It has previously been reported by Wenemoser and Reddien [2010] that planarian regeneration subsequent to tissue wounding progresses with two distinct peaks of mitotic activity. The first peak occurs following a decrease in mitotic activity at approximately 45 min. While this would seem to be contradictory to our results, we suggest that the exposure to the EMF inverted this natural effect of decreased mitotic activity. This mitotic peak is reportedly a fivefold increase in activity [Wenemoser and Reddien, 2010] and has been linked to an activation of the signaling pathways of the G2/M transition phase.

The G2/M transition pathway has previously been reported to be activated following exposure to ionizing radiation, the signaling chain responsible for this effect being the p38-mitogen activated protein kinase pathway (MAPK) [Bulavin et al., 2001]. Furthermore, this same pathway has been shown to be activated by non-ionizing radiations associated with cellular telephones [Leszczynski et al., 2012] as well as the hsp27 pathway. These experiments, in addition to those of Goodman et al. [2009] indicate that low frequency EMFs can activate the hsp70 extracellular signal-regulated kinase pathway (ERK). This supports our hypothesis that the 30–45 min inflection point observed in the present study is a result of the aforementioned biochemical sequences. These pathways are all associated with an increase in cell proliferation as well as being intimately involved with the G2/M transition phase.

CONCLUSION

While definite signaling pathways have yet to be elucidated, the present study has demonstrated that specialized patterned EMFs are capable of increasing the rates of planarian regeneration after exposure of only 30–45 min. This duration coincides with a natural decrease in mitotic activity, which we hypothesize is countered by the EMF exposure. That a number of known biochemical pathways involved with cell proliferation have been shown to be activated by exposure to ionizing and non-ionizing radiations suggests that these are the biological mechanisms through which the "Thomas" field has its effect.

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